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MICROSATELLITE DNA MARKER AIDED DIVERSITY ANALYSIS IN CONFECTIONERY SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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ABSTRACT

Genetic diversity among 48 genotypes of confectionery sunflower (Helianthus annuus L.) collected from USDA, USA and India were evaluated by using microsatellite SSR markers. Twenty five sunflower specific SSR primers were used. Of the 25 SSR primers used, 10 primer pairs (ORS331, ORS694, ORS728, ORS785, ORS807, ORS878, ORS 378, ORS1265,ORS1265,ORS1242) showed polymorphism. The high level of polymorphism (66.66%) was reported in this finding and the number of alleles in SSR loci ranged from 2 to 4 with an average of 2.5. The present study identifies the promising lines EC 734807, EC 734808,EC 734810,EC734860 and EC 734817 for protein and yield, these can be used as potential donors in future hybridization programme.

KEYWORDS: Helianthus Annuus Molecular Diversity SSR Markers

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Original Article

INTRODUCTION

Sunflower (*Helianthus annuus* L.) as a source of vegetable oil and protein is grown in many parts of the world. It is the fourth important vegetable oil crop (Vollmann and Rajcan, 2009), producing 9 million tonnes per year. It is grown in all over the world with three main purposes: ornamental, oilseed and confectionery sunflower. Confectionery sunflower produces large seeds with low oil content and it is used in baking and snack applications. Growing confectionery sunflower (*Helianthus annuus* L.) for consumption is becoming more and more attractive in the whole world. The prime aims of the confectionery sunflower is to improve the seed yield with greater content of protein in seed, increased mass of 1000 seed, and thin hull being separated easily from the kernel, so that it is very convenient for mechanical nibbling and further processing of the kernel. Plant genetic resources are of paramount importance for the future and to ensure the food and nutritional security of an increasing population (Choudhury*et al.* 2014). So, estimating genetic diversity and determining the relationships in germplasm collections enhances efficient germplasm management and genetic improvement. DNA markers can be used as simple, accurate, and rapid way for the evaluation of genetic diversity and grouping of individuals in oily (Vukich*et al.* 2009; Zia *et al.* 2014) and confectionery sunflower (Dong *et al.* 2007; Kholghi*et al.* 2012) germplasm. Review of literature showed very limited works on the predication of confectionery sunflower heterosis and hybrid performance by marker based genetic distance of the parental lines (Tersac*et al.*, 1994; Chere*set al.*, 2000). The purpose of this study was to determine the potential use of SSR markers in confectionery sunflower and evaluate the level of genetic variation among 47 confectionery sunflower Germplasm lines collected from USDA,USA.

MATERIALS AND METHODS

The materials used in the present study consisted of 47 germplasm accessions of confectionery sunflower (*Helianthus annuus* L.) obtained from the United States Department of Agriculture (USDA), United States of America along with one check (Surya). The list of confectionery sunflower genotypes along with one check Surya used for study are listed in the Table 1. These genotypes were of diverse in nature, both with respect to geographical distribution, yield and protein components. The standard level of protein and oil content in hybrids/varities varies from 36-38% and 34-36% respectively. Surya was usedas check along with the confectionery sunflower germplasm lines. All the germplasm lines were raised in a simple lattice design in the field during summer, 2014. A spacing of 60 x 30 cm was adopted, with standard agronomic practices followed throughout the period of crop growth. Young healthy leaves were collected and pooled from 2-3 weeks old field grown confectionery sunflower genotypes, washed free of dirt, mopped dry and quickly frozenand powdered using liquid nitrogen. The samples were used for immediate isolation of DNA. DNA was isolated from each genotype as per the standard procedure (Dellaporta*et al*., 1983). SSR Primers: The 25 primers used in this study were preselected sunflower genomic SSR's based on their performance with sunflower DNA in earlier studies. The choice of SSR markers was based on clarity of produced bands and their genetic locations in order to give a uniform coverage of the sunflower genome (PoormohammadKiani*et al.* 2007; Tang *et al.* 2002). Primer names, their sequences and annealing temperature are listed in Table 2.

Microsatellite marker analysis was carried out with primers obtained from GeNei. Polymerase chain reaction (PCR) and agarose gel electrophoresis: The PCR reaction mixture consisted of 2.0µl DNA, 10.8µl of sterile water, 2.0µl Buffer (10X), 0.6µl dNTP's (10mM), 2.0µl Mgcl (25mM),1.0 µl Primer Forward (5pM), 1.0µl Primer Reverse (5pM) and 0.6µl Taq polymerase. Temperate DNA was initially denatured at 94^0C for 5 min, followed by 38 cycles of 94^0C for 1 min denaturation, primer annealing temperature between 52^0C to 68.5^0C for1min and 2 min primer extension at 72^0C. Final 5 min incubation at 72^0C was allowed for completion of primer extension on an Eppendorf thermal cycler. The amplified products were electrophoretically resolved on 3 % agarose in 1 x TAE buffer (Don *et al*., 1991). Gel scoring and data analysis: Each band or fragment was tested as a separate putative locus and were scored using binary mode with "0" indicating the absence and "1" indicating presence of band. This binary dataset were used to calculate pair-wise similarity coefficient by simple matching (SM) coefficient method using SIMIQUAL program. The matrix of similarity coefficient was subjected to unweighted pair group method using arithmetic mean (UPGMA) to generate a dendrogram using average linkage procedure. All the numerical analysis was performed using the computer program NTSYS-pc-version 2.0.

RESULTS AND DISCUSSIONS

The objective of the present study was to assess the extent of genetic diversity and relationships among 48 confectionery sunflower genotypes. The confectionery sunflower genotypes were analyzed by using 25 SSR primers. Twenty five sunflower specific SSR primers were used. Of the 25 SSR primers used, 10 primer pairs (ORS331, ORS694, ORS728, ORS785, ORS807, ORS878, ORS 378, ORS1265,ORS1265,ORS1242) showed polymorphism. The number of alleles produced by different primers ranged from two to four. The SSR profile for 48 confectionery sunflower genotypes generated by primer ORS 378 and ORS 1265 are shown in Fig. 1and 2. The high level of polymorphism (66.66%) was reported in this finding and was supported by the earlier workers,Carla V Filippi*et al.* (2015) and MarjanJannatdoust*et.al* (2016). MarjanJannatdoust*et.al* (2016) reported that the high level of microsatellite polymorphism within confectionery sunflower population (70%) compared to between populations (30 %).

In the present study, SSR marker system showed high dissimilarity among the confectionery sunflower genotypes. Higher the dissimilarity between the genotypes, better the scope to include them for breeding programme. The genotypes EC-734821,Surya and EC 734790 had the highest dissimilarity value which indirectly showed the extent of genetic diversity exist among these genotypes. Cluster diagram constructed using polymorphic markers identified eight major clusters (Fig.3). Each cluster comprises of 2 to 3 sub clusters. All the high protein genotypes Viz., EC 734808,EC 734800 and EC 734792 were grouped under one sub cluster. Whereas the genotypes EC 734810,EC 734879 and EC 734817 with high protein were grouped under three sub clusters. The genotype EC734807 recorded high yield with higher protein percentage and kernel weight grouped under different sub cluster. However, there was variation in the composition of genotypes in sub groups. The genotypic similarity might be due to same geographical origin. The clustering based on different characters and according to the geographical location from where they were collected was reported by Urs and Uma,2016. The protein content contributed more towards the genetic divergence.

However some low protein genotypes were grouped in same cluster with moderate to high yielders in sub clusters. Grouping together of genotypes in the same cluster irrespectively of their geographical origin indicates the genetic uniformity produced through artificial selection. The sub clusters containing one genotype each (EC-734851, EC-734821 EC-734837and EC-734844 respectively), indicating the higher divergence of these genotypes. Genotypes from distinct clusters could be used in hybridization program to get high protein and high yield varieties. In conclusion, the results also reveal wide polymorphism indicating the available variability at genetic level. Higher polymorphism/variation was observed in genotypes. Therefore, the use of genes of these genotypes needs to be exploited further and can be used in breeding programs. The genetic diversity obtained in this study might be useful in future strategies for evaluation of desired genotypes. Such molecular data would be also useful for detecting DNA patterns unique for a given accession or set of accessions. Finally, our results demonstrate the feasibility of the SSR markers for quantifying genetic distances among 48 confectionery sunflower genotypes. The present study indicates inclusion of EC 734807, EC 734808,EC 734810,EC734860 and EC 734817 as potential donors for future hybridization programme would result in the development of superior confectionery sunflower cultivars.

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Table 1: Qualitative Traits of Confectionery Sunflower Germplasm Linesused in the Study

Sl. No	Accession Number	Seed Colour	Seed Size	Seed Shape	Plant Height	Protein Content %
1.	EC 734790	Black, white-stripes	Large	Elongated	Tall	17.69
2.	EC 734791	Black, brown-stripes	Large	Elongated	Medium	21.71
3.	EC 734792	Grey, white-stripes	Medium	Bulged	Tall	25.78
4.	EC 734795	White grey stripes	Medium	Pointed	Tall	18.32
5.	EC 734800	Grey, white-stripes	Medium	Pointed	Tall	26.43
6.	EC 734855	Grey, white-stripes	Medium	Elongated	Medium	19.83
7.	EC 734851	Black, white-stripes	Medium	Pointed	Tall	22.58
8.	EC 734803	White	medium	pointed	Tall	19.68
9.	EC 734804	Grey, white-stripes	Medium	Bulged	Tall	24.06
10.	EC 734805	Grey, white stripes in boarder	Medium	Elongated	Tall	23.32
11.	EC 734806	Grey, white-stripes	Medium	Pointed	Tall	20.37
12.	EC 734807	Grey, white-stripes	Medium	Pointed	Tall	27.50
13.	EC 734808	Black	Medium	Bulged	Tall	30.38
14.	EC 734809	Grey, white-stripes	Medium	Broad	Medium	19.63

Sl. No	Accession Number	Seed Colour	Seed Size	Seed Shape	Plant Height	Protein Content %
15.	EC 734810	Brown, white-stripes	Medium	Broad-pointed	Medium	30.15
16.	EC 734813	White	Medium	Elongated	Tall	20.15
17.	EC 734814	Brown, white stripes	Medium	Broad-pointed	Medium	18.74
18.	EC 734815	White, grey stripes	Medium	Pointed	Dwarf	21.80
19.	EC 734816	Grey, white stripes in boarder	Medium	Broad-pointed	Dwarf	18.06
20.	EC 734817	Grey, white stripes in boarder	Medium	Elongated	Medium	24.70
21.	EC 734818	Grey, white stripes in boarder	Medium	Bulged	Medium	22.30
22.	EC 734820	Grey, white-stripes	small- Medium	Broad	Dwarf	22.23
23.	EC 734821	Grey, white-stripes	Medium	Elongated	Dwarf	18.58
24.	EC 734823	Grey, white-stripes	Large	Broad	Medium	20.30
25.	EC 734824	Grey	small- Medium	Broad-pointed	Dwarf	21.91
26.	EC 734827	Grey, white-patch	Medium	Pointed	Dwarf	19.49
27.	EC 734828	Grey, white-stripes	small- Medium	Pointed	Dwarf	23.51
28.	EC 734831	Grey, white-stripes	Medium	Slender	Medium	21.81
29.	EC 734833	Light grey, white-patch	Medium	Broad	Medium	22.90
30.	EC 734835	Grey, white stripes in boarder	small- Medium	Broad	Tall	22.53
31.	EC 734837	Grey, white-stripes	Medium	Elongated	Dwarf	22.85
32.	EC 734842	Light brown, grey stripes	Small	Pointed	Medium	21.07
33.	EC 734844	Grey, white-stripes	small- Medium	Pointed	Tall	19.78
34.	EC 734846	Black	Medium	Bulged	Tall	21.21
35.	EC 734853	Grey, white-stripes	Medium	Pointed	Medium	22.18
36.	EC 734854	Brown, white-stripes	Medium	Bulged	Tall	21.91
37.	EC 734860	Grey, white-stripes	Medium	Elongated	Tall	23.18
38.	EC 734863	Grey, white-stripes	Medium	Broad	Medium	21.69
39.	EC 734865	Grey, white-patch	Medium	Broad-pointed	Medium	21.11
40.	EC 734866	Grey, white-stripes	Medium	Broad	Dwarf	20.33
41.	EC 734867	Grey, white-stripes	Medium	Broad-pointed	Dwarf	21.71
42.	EC 734869	Black, white- stripe in boarder	small- Medium	Broad-pointed	Dwarf	21.78
43.	EC 734879	Grey, white stripes in boarder	Medium	Broad-pointed	Dwarf	25.55
44.	EC 734883	Grey, white-patch	small- Medium	Oval	Dwarf	22.82
45.	EC 734884	Grey, white-stripes	Medium	Pointed	Dwarf	21.25
46.	EC 734885	Grey, white-stripes	Medium	Elongated	Dwarf	21.58
47.	EC 734799	Grey, white stripes in boarder	Larger	Pointed	Medium	22.47
Standard						
48.	Surya	Grey, white stripes	Small	Broad Pointed	Medium	18.21

Table 2: List of Sunflower SSR Primers used, their Sequences and Annealing Temperature

Sl No	Primer Name	Forward Sequence and Reverse Sequence	Annealing Temperature ^{0}c	Mean Temp In ^{0}c	GC Ratio In %
1	ORS 94 F	TGCAAGGTATCCATATTCCACAA(23)	57.1	57.7	39.1
	ORS 94 R	TATACGCACCGGAAAGAAAGTC(22)	58.4		45.5
2	ORS 216 F	TCCCTAATGTACCACCACCATC(22)	60.3	60.6	50
	ORS 216 R	CTTCCTCCACCCTCAAGCG(19)	61.0		63.2
3	ORS 240 F	CACTCAACCATTGTTCTCCCAC(22)	60.3	61.2	50
	ORS 240 R	GGTGATGATGGAGGAGCAACTG(22)	62.1		54.5
4	ORS 254 F	CCTTCAGTGCTCATGCAGTG(20)	59.4	57.05	55
	ORS 254 R	AAATCCCACTTCATACAAACGT(22)	54.7		36.4
5	ORS 258 F	TTGCGTCCGATGCTGTTC(18)	56	56.6	55.6
	ORS 258 R	GGCCCGATTACAAGATAACG(20)	57.3		50
6	ORS 261 F	AGCGAAAGGATCGAGAATCATC(22)	58.4	59.3	45.5
	ORS 261 R	CTGTTCCGTTCGTCAGAAACTC(22)	60.3		50
7	ORS 331 F	TGAAGAAGGGTTGTTGATTACAAG(24)	57.6	56.4	37.5
	ORS 331 R	GCATTGGGTTCACCATTTCT(20)	55.3		45
8	ORS 378 F	GTGAAACCTTCGGACCTCTG(20)	59.4	55	55
	ORS 378 R	GTACAAAACTTATAAATAAAACAATA(26)	50.6		15.4
9	ORS 488 F	CCCATTCACTCCTGTTTCCA(20)	57.3	57.3	50
	ORS 488 R	CTCCGGTGAGGATTTGGATT(20)	57.3		50
10	ORS 608 F	CATGGAAAGCCGAGTTCTCT(20)	57.3	57.3	50
	ORS 608 R	CGTGCGTGATTAACATACCC(20)	57.3		50
11	ORS 609 F	GCGAAGGAACTGAACCGTATA(20)	57.3	56.3	50
	ORS 609 R	GGATTTTAGTCCGCCAATCA(20)	55.3		45
12	ORS 621 F	CGCCTTATGCTGAGAGGAAA(20)	57.3	57.3	50
	ORS 621 R	CCTGAAGCGAAGAAGAATCG(20)	57.3		50
13	ORS 694 F	CCTGGAACTGAACCGAGAAC(20)	59.4	59.4	55
	ORS 694 R	GCCGTGAAACAGAGAGAGGA(20)	59.4		55
14	ORS 718 F	CACTTTACGCACACCAAACC(20)	57.3	56.3	50
	ORS 718 R	ATGCAACACCCGAATCAAAG(20)	55.3		45
15	ORS 728 F	CTCCATAGCAACCACCTGAAA(21)	57.9	58.8	47.6
	ORS 728 R	CCAAACTCTGAATGATACTTGTGAC(25)	59.7		40
16	ORS 785 F	CAAAATACCCAGGTCAAAGCA(21)	55.9	58.1	42.9
	ORS 785 R	CCTAGCTTATGGGACGTATGGA(22)	60.3		50
17	ORS 807 F	CCGATATTTTGACCGATATTTTGC(24)	57.6	58.7	37.5
	ORS 807 R	TCTCACCCTTCATCTCCTTCC(21)	59.8		52.4
18	ORS 844 F	ACGATGCAAAGAATATACTGCAC(23)	57.1	57.2	39.1
	ORS 844 R	CATGTTTAATAGGTTTTAATTCTAGGG(27)	57.4		29.6
19	ORS 878 F	CCAAAGGTGGGATAACCTAAAAG(23)	58.9	56.1	43.5
	ORS 878 R	TCCGTGTTCATGCATTGATT(20)	53.2		40
20	ORS 996 F	CGGTGAGAATAACCTCGGAAGA(22)	60.3	59.6	50
	ORS 996 R	ATCAGTCCTTCAACGCCATTAGT(23)	58.9		43.5
21	ORS 1079 F	TACGACTGACGATTCCATTTCTC(23)	58.9	58.9	43.5
	ORS 1079R	AACTGGATTTCAGGGAGTGTT(23)	58.9		43.5
22	ORS 1179 F	GATTCGGAGCTGTTAGGAGGTAG(23)	62.4	59.8	52.2
	ORS 1179R	AAACGGGAAGCAAGAATAGAACA(23)	57.1`		39.1
23	ORS 1215 F	ATACTCTTCCACCCTCAAATCCA(23)	58.9	61.0	43.5
	ORS 1215R	GGTTGCGGTAGTGGTCTGTAGT(22)	62.1		54.5
24	ORS 1242 F	GCAATCGTTTCACTCTTCCATTC(23)	58.9	58.6	43.5
	ORS 1242R	TGGTCGTAGAATTGTCGGTCAT(22)	58.4		45.5
25	ORS 1265 F	GGGTTTAGCAAATAATAGGCACA(23)	57.1	57.5	39.1
	ORS 1265R	ACCCTTGGAGTTTAGGGATCA(21)	57.9		47.6

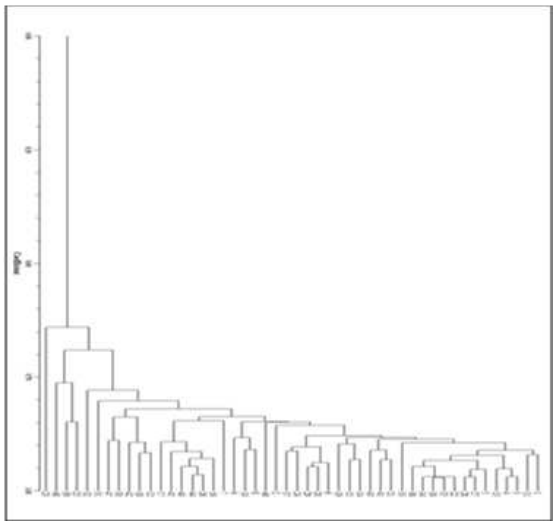

Figure 3: Molecular Dendrogram of 48 Confectionery Sunflower Germplasm Lines Constructed with SSR Primers

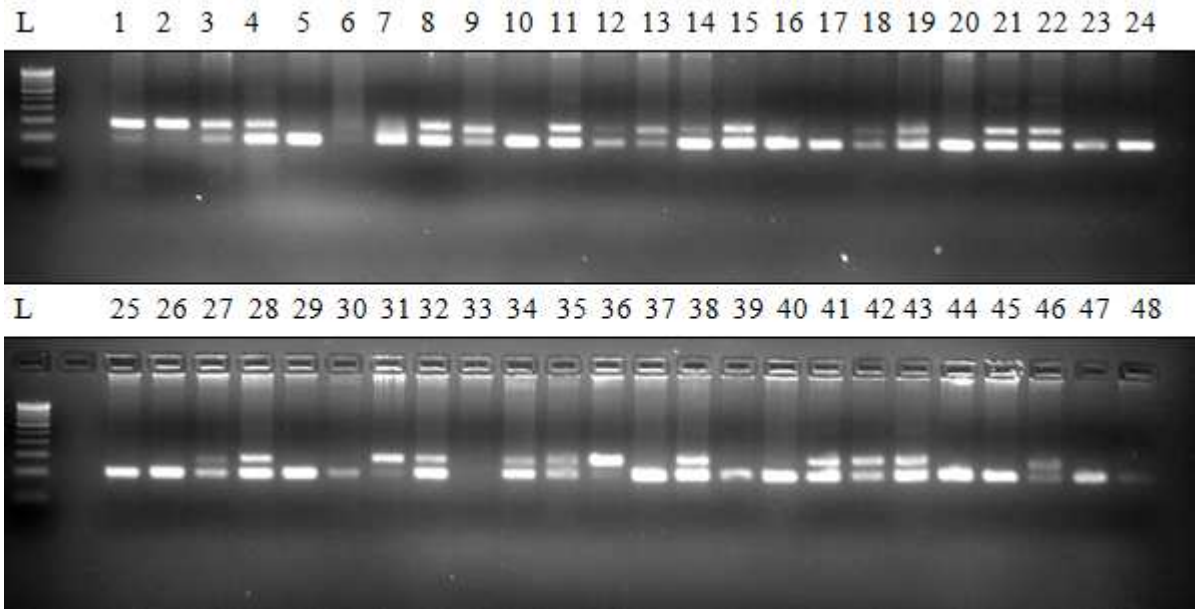

Figure 1: DNA Amplified Product of ORS 378 for 48 Confectionery Sunflower Germplasm Lines Resolved on Agarose Gel

Primer ORS 378, M: Molecular weighted marker (100bp),1 to 48 : Confectionery sunflower germplasm lines

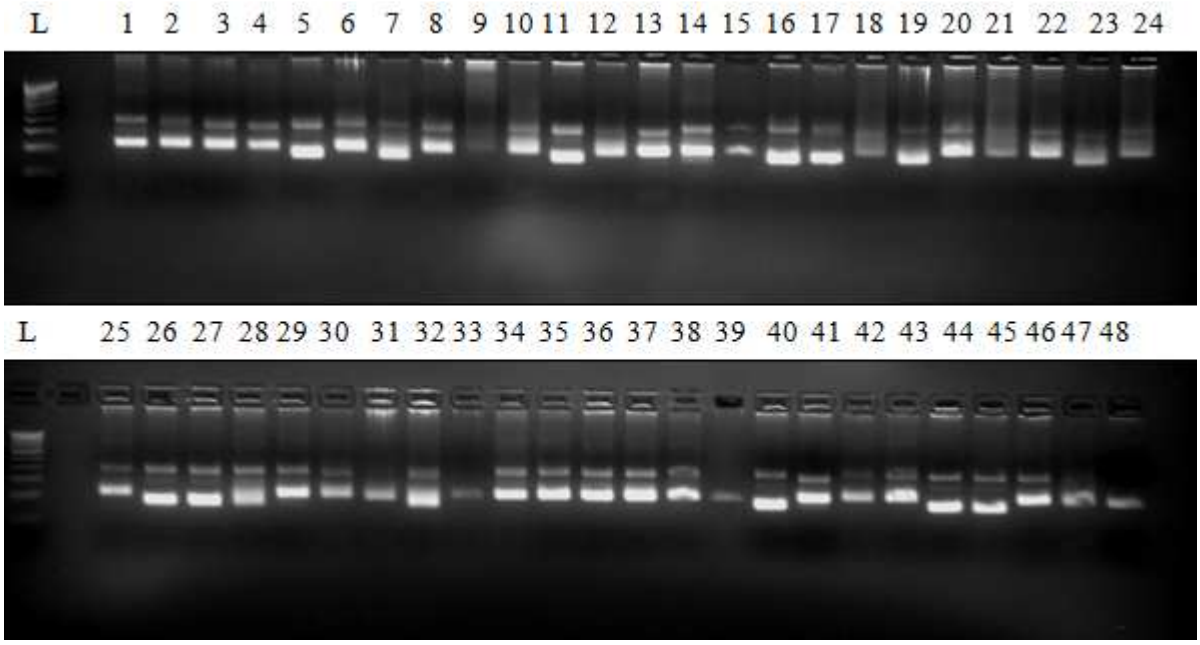

Figure 2: DNA Amplified Product of ORS 1265 for 48 Confectionery Sunflower Germplasm Lines Resolved on Agarose Gel

Primer ORS 1265 M: Molecular weighted marker (100bp),1 to 48 : Confectionery sunflower germplasm lines